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Purification, characterization and safety assessment of the introduced cold shock protein B in DroughtGard™ maize

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ABSTRACT

DroughtGard™ maize was developed through constitutive expression of cold shock protein B (CSPB) from *Bacillus subtilis* to improve performance of maize (*Zea mays*) under water-limited conditions. *B. subtilis* commonly occurs in fermented foods and CSPB has a history of safe use. Safety studies were performed to further evaluate safety of CSPB introduced into maize. CSPB was compared to proteins found in current allergen and protein toxin databases and there are no sequence similarities between CSPB and known allergens or toxins. In order to validate the use of *Escherichia coli*-derived CSPB in other safety studies, physicochemical and functional characterization confirmed that the CSPB produced by DroughtGard™ possesses comparable molecular weight, immunoreactivity, and functional activity to CSPB produced from *E. coli* and that neither is glycosylated. CSPB was completely digested with sequential exposure to pepsin and pancreatin for 2 min and 30 s, respectively, suggesting that CSPB will be degraded in the mammalian digestive tract and would not be expected to be allergenic. Mice orally dosed with CSPB at 2160 mg/kg, followed by analysis of body weight gains, food consumption and clinical observations, showed no discernible adverse effects. This comprehensive safety assessment indicated that the CSPB protein from DroughtGard™ is safe for food and feed consumption.

1. Introduction

Water availability is the most important factor limiting crop productivity in dry and semi-dry land areas (Castiglioni et al., 2008). It has been demonstrated that the expression of bacterial cold shock proteins (CSPs) in plants can confer improved drought stress adaptation in multiple plant species (Castiglioni et al., 2008). To improve performance of maize (*Zea mays*) under water-limited conditions, expression of the gene encoding cold shock protein B (CSPB) has been used to develop a drought-tolerant maize, DroughtGard™ (Castiglioni et al., 2008; Harrigan et al., 2009). CSPB, from the bacterial species *Bacillus subtilis*, belongs to the family of single-stranded nucleic acid binding proteins that contain a highly conserved cold shock domain (CSD) (Zeeb and Balbach, 2003). Structurally similar to other bacterial CSPs, CSPB is composed of five antiparallel β-strands forming a five-strand β-barrel, and possesses two conserved RNA binding ribonucleoprotein motifs found in other RNA-binding proteins (Bienert et al., 2004; Manival et al., 2001). Functionally, CSPs are generally considered to help cell survival at low temperatures by destabilizing RNA secondary structures (Graumann et al., 1997; Max et al., 2006; Mussgnug et al., 2005; Phadtare and Inouye, 1999; Phadtare et al., 2002; Yamanaka et al., 1998). In bacteria, some CSPs rapidly accumulate in response to temperature reduction, while others, including CSPB, are present not only in low temperature conditions but also under optimal growth conditions and after entry into the stationary phase (Graumann et al., 1997). Additionally, CSPs are involved in maintaining normal cellular functions under nutrient limitation (Anderson et al., 2006).

CSD-containing proteins have also been identified in various plant species including rice, wheat and barley (Chaikam and Karlson, 2008; Karlson and Imai, 2003). *B. subtilis*, the source of the *cspb* gene inserted into DroughtGard™ maize through recombinant DNA methods, is a microorganism with a history of safe use in fermented foods and probiotics (De Boer and Diderichsen, 1991; Hosoi et al., 2003; Sanders et al., 2003). *B. subtilis* is used in production of thua nao, popular in Asian food as a condiment for enhancing flavor in soups and curries, and natto, a commonly consumed food in Japan for hundreds of years, made by fermenting cooked

Abbreviations: BSA, bovine serum albumin; CSPB, cold shock protein B from *Bacillus subtilis*; CSD, cold shock domain; DLP, Dual Labeled Probe; *E. coli*, *Escherichia coli*; HRP, horseradish peroxidase; MALDI–TOF MS, matrix assisted laser desorption ionization–time of flight mass spectrometry; MOE, margin of exposure; NOAEL, no observable adverse effect level; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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soybeans with *B. subtilis ssp. natto* (Ashikaga et al., 2000; Inatsu et al., 2006). In addition, *B. subtilis* is Generally Recognized As Safe (GRAS) by the US Food and Drug Administration (U.S. FDA) for use in the manufacturing of enzyme preparations to be used in food (FDA, 1999; OECD, 2001). These common uses support the conclusion that *B. subtilis* is safe for human consumption (Pedersen et al., 2002; Sorokulova et al., 2008).

Due to the food uses of *B. subtilis* and other CSP-containing bacteria, as well as of plant proteins with CSDs, a history of safe exposure for this protein is well established. Therefore, according to Codex guidance and the tiered approach advocated by the International Life Sciences Institute, there is no safety concern for CSPB consumption (Codex Alimentarius, 2009; Delaney et al., 2008a; Hammond et al., 2013). However, to explicitly assess whether the CSPB introduced into maize is safe for food and feed consumption, safety assessments of this protein were performed prior to commercialization of DroughtGard™. Here we describe processes for evaluating the safety of CSPB, which was according to guidance documents (Codex Alimentarius, 2009; Gao et al., 2004; Harrison et al., 1996; Herouet et al., 2005; Raybould et al., 2013).

2. Materials and methods

2.1. Purification of CSPB from Escherichia coli

The deduced amino acid sequence of the *cspb* gene expressed in DroughtGard™ is identical to that of the *cspb* gene from *B. subtilis*, with the exception of a substitution that results in valine, rather than leucine, at position 2 of the deduced sequence. The appropriate *cspb* coding sequence was ligated into pET24b (Novagen, Madison, WI) and expressed in BL21 (DE3) *E. coli* (Invitrogen, Carlsbad, CA). Fermentation was performed with 240 L of a medium containing Tryptone 10 g/L, Yeast extract 5 g/L, sodium chloride 5 g/L and Kanamycin 50 mg/L. The CSPB protein expression was induced by addition of isopropyl-1-thio-β-galactopyranoside to a final concentration of 0.5 mM at 37 °C overnight. The *E. coli* cell paste was resuspended in 50 mM Tris, pH 8.0, 10 mM KCl and 1 mM EDTA extraction buffer at approximately a 1:5 sample weight to buffer volume ratio. Cells were disrupted by a microfluidizer followed by incubation with Benzonase at ~20 units/ml. The cell lysate was clarified by centrifugation. Ammonium sulfate was slowly added to the clarified extract to a final saturation of 40%. After centrifugation, the supernatant was loaded onto a Phenyl Sepharose 6 Fast Flow column (All chromatography resins were from GE Healthcare, Piscataway, NJ) and eluted with a linear salt gradient that decreased from 1.7 to 0 M $(NH_4)_2SO_4$ in the 50 mM phosphate pH 7.0 buffer. After dialysis, the pooled CSPB containing fraction was then loaded on a Q Sepharose Fast Flow column and eluted with a linear salt gradient that increased from 0 M to 0.5 M NaCl in a buffer of 20 mM Tris, pH 8.0 over 10-fold bed volumes. The CSPB-containing fractions were pooled and concentrated using dialysis tubing (Spectrum laboratories, Inc, Rancho Dominguez, CA) [Molecular Weight Cut Off (MWCO): 3500] against Aquacide I. This concentrated CSPB sample was further polished using a size exclusion column (S100, Sephacryl, GE Healthcare, Piscataway, NJ) equilibrated and eluted with a buffer of 20 mM Tris, pH 8.0. CSPB-containing fractions were pooled as the final fraction.

2.2. Purification of CSPB from grain of DroughtGard™ maize

CSPB protein was extracted from 10 kg of the grain powder of DroughtGard™ with 100 L of Tris–Borate buffer (89 mM Tris–Borate, 2 mM EDTA, pH 8.3) and incubated overnight at ~4 °C with constant stirring. Diatomaceous earth (Advanced Minerals Corp, Goleta, CA) was added to a final concentration of 7.5% (w/v) and mixed for 3 h prior to filtration. The final slurry was filtered using an Ertel Alsop filter press (Kingston, NY) with Die 42 micro media filter pads and a Cuno filter (Hagedorn & Gannon Co., Inc.). The filtrate was then concentrated by diafiltration using a polysulfone hollow fiber cartridge with a 3000 MWCO (GE Healthcare, Piscataway, NJ). Ammonium sulfate was slowly added to the concentrated extract to a final saturation of 40% and was allowed to dissolve overnight at 4 °C. After centrifugation, the pellet was discarded and the supernatant collected and diafiltrated against buffer A (20 mM Tris, pH 7.0),

The diafiltrated sample was separated by a Q Sepharose Fast Flow column as described above. The CSPB-containing fractions were pooled and concentrated using diafiltration as described above. The concentrated sample containing CSPB was re-circulated over an immunoaffinity column utilizing a mouse anti-CSPB monoclonal antibody (raised against the *E. coli*-expressed CSPB protein) immobilized onto AminoLink resin (Pierce, Rockford, IL). Bound CSPB was eluted using 100 mM triethylamine buffer, pH 11 and neutralized with 1/20th volume of 1 M sodium phosphate, pH 6.8. The process was repeated multiple times until most of CSPB present in the pool was captured. This immunoaffinity-enriched CSPB fraction was further polished by size exclusion chromatography as described above using buffer A. The CSPB containing fractions were pooled and concentrated sequentially by diafiltration with a mini cartridge (GE Healthcare, Piscataway, NJ) and by dialysis (Slide-A-Lyzer dialysis cassette MWCO: 3500, size: 0.5–3 ml, Pierce, Rockford, IL) against Aquacide I.

2.3. Protein characterization

Purity and apparent molecular weight of CSPB were determined using densitometric analysis of stained SDS–PAGE gels.

For immunoblot analysis, plant- and *E. coli*-produced CSPB were subjected to SDS–PAGE and transferred to a nitrocellulose membrane. The blot was probed with a goat anti-CSPB specific polyclonal antibody raised against the *E. coli*-expressed CSPB protein.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS) was used to confirm the identity of CSPB by tryptic digest mapping (Aebersold, 1993; Billeci and Stults, 1993; Hileman et al., 2006). N-terminal sequence analysis (Hunkapiller and Hood, 1983) was performed for 15 cycles using an Applied Biosystems 494 Procise Sequencing System. To determine whether CSPB was glycosylated, proteins on a PVDF membrane were analyzed using a Molecular Probes' Pro-Q® Emerald 488 Glycoprotein Blot Stain Kit (Invitrogen, Carlsbad, CA). Following glycosylation analysis, total protein on the membrane was visualized using a SYPRO® Ruby Protein Blot Stain kit (Invitrogen, Carlsbad, CA).

A CSPB functional activity assay was performed to analyze the ability of CSPB to unfold an oligonucleotide-hairpin structure (Phadtare et al., 2003, 2004). Briefly, the CSPB activity was assessed as the amount (pmol) of unfolded Dual Labeled Probe (DLP) per microgram of CSPB. The DLP consists of a synthesized 35-base oligonucleotide fragment with a fluorescent label at the 5′ end and a fluorescence quencher at the 3′ end. The DLP was designed to form a double stranded stem of six base pairs due to the complementary bases located at 5′ and 3′ ends, while 23 nucleotides (dT) in the middle form a loop. The fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The amount of opened DLP was determined using a calibration curve of the fluorescent tag.

2.4. Bioinformatic assessment of potential CSPB allergenicity and toxicity

The CSPB protein sequence was translated from its corresponding nucleotide sequence using standard genetic code. FASTA

analyses evaluated the similarity of the CSPB protein sequence to sequences in allergen (FARRP, 2014), protein (GenBank, release 199.0) and protein toxin databases. All protein sequences (32,476,608) in GenBank Release 199 were downloaded and the description line for each sequence was searched to see if it contained the keywords "toxi" or ricin. Sequences (35,473) containing the keywords were identified; each description line was reviewed and sequences having descriptions that were consistent with protein toxicity were retained. Sequences whose descriptions did not reflect toxicity, i.e. anti-toxin, non-toxin, toxin export etc. were discarded. Those sequences that remained were screened to remove duplicates resulting in 10,419 sequences that comprised the TOX_2014 database. Each protein alignment received an *E*-score, which is a composite measure of similarity between proteins that were aligned by FASTA. When an *E*-score cutoff of 1 was used, no alignments were obtained with the TOX_2014 database. By definition a FASTA alignment with an *E*-score of 1 is what would be observed for random sequence of the same length and amino acid composition as CSPB. When the *E*-score threshold was raised to 10, 96 alignments were recovered. Among the top 5 alignments that displayed an *E*-score greater than one and less than two, three classes of toxins were identified; they include RTX toxins, parasporins and phage toxin ribonucleases. These alignments spanned no more than 42 amino acids; they displayed no greater than 40% identity and each of the aligning toxins from the database was greater than 598 amino acids in length. Overall, the alignments were of exceptionally low quality and did not indicate sufficient conservation in structure such that one would conclude that CSPB would display the function of an RTX toxin, a parasporin or a phage toxin ribonuclease.

An *E*-score of $\leqslant$1e−5 (1×10^{-5}) was set as an initial high cut-off value for alignment significance and represents a conservative and inclusive measure for the identification of proteins that are likely to be homologous (Pearson, 2003). In addition, the allergen database was screened for matches using an eight amino acid sliding window across the CSPB sequence (Goodman et al., 2002; Hileman et al., 2002; Silvanovich et al., 2006; Stadler and Stadler, 2003). While typically FASTA alignments with sequences from the allergen database are examined to see if they exceeded a threshold of 35% identity in 80 amino acids (Codex Alimentarius, 2009), this was criterion not applied due to the fact that the CSPB protein is smaller than 80 amino acids. Instead, all FASTA alignments with the allergen database were inspected to confirm that no alignment displayed equal to or greater than 28 identities, the number needed in 80 amino acids to yield 35% identity. The maximum number of identities observed in any alignment with the allergen database was 17 spread over an alignment window of 50 amino acids. As with the toxin alignments, the quality of alignments with the allergen database was so poor that there was no indication of conserved structure between CSPB and any allergen sequence.

2.5. Assessment of CSPB susceptibility to pepsin and pancreatin

The susceptibility of the *E. coli*-produced CSPB to pepsin was evaluated using a previously described method (Thomas et al., 2004). Prior to treatment, the specific activity of pepsin (Sigma Company, St. Louis, MO) was quantified. CSPB was mixed with pepsin solution (10 mM HCl, 2 mg/ml NaCl, pH ~1.2; 10 U of pepsin per microgram of CSPB protein) and incubated at 37 °C. Samples were removed at 0.5, 2, 5, 10, 20, 30 and 60 min and quenched with a buffer of 0.7 M Na_2CO_3, pH 11. The resulting solutions were analyzed by SDS–PAGE.

Cleavage by digestive enzymes was also assessed by pepsin treatment followed by pancreatin treatment. Pancreatin (Sigma) was dissolved in 50 mM potassium phosphate buffer (pH 7.5) to 1% (w/v) as described in The United States Pharmacopoeia (Delaney et al., 2008b; USP, 1995). The pancreatin solution was formulated so that 55.3 μg of pancreatin powder would be present per μg of the CSPB protein in the reactions. The pepsin reaction was halted at 2 min of incubation by adding 0.7 M sodium carbonate buffer; the 2 min time point was selected to insure that the protein fragment of interest was readily detectable on SDS–PAGE. The quenched pepsin reaction mixture was added to a pre-heated (37 °C, 5 min) aliquot of pancreatin mixture. Samples were removed at 0, 0.5, 2, 5, 10, 30, 60 and 120 min and quenched with SDS–PAGE loading buffer. The resulting solutions were analyzed by SDS–PAGE.

2.6. Acute oral toxicity assessment

CD-1 mice (Charles River Laboratories, Spencerville, OH) were acclimated for 7 days before the first day of dosing. Prior to study start the animals were weighed and examined in detail, and then assigned to the study utilizing a stratified randomization technique to obtain approximately equivalent group mean body weights for the two treatment groups (each comprised of 10 male and 10 female CD-1 mice). The animals were approximately 8 weeks of age at the initiation and their body weights ranged from 28.8 to 35.2 g for the males and 21.2 to 25.2 g for the females.

The CSPB dose solution was prepared by mixing *E. coli*-derived CSPB in a vehicle buffer (20 mM Tris, pH 8.0) at a dose concentration of 61.7 mg/ml. A control dose solution was prepared by mixing lyophilized BSA powder (Calbiochem, La Jolla, CA) with the vehicle buffer to achieve a similar protein concentration to CSPB. Pre-dose, post-dose, and homogeneity samples were collected for analysis. After dilution, the pre- and post-dose samples were assessed for CSPB purity and apparent molecular weight using SDS–PAGE, for protein concentration using amino acid analysis, and functional activity of the protein using the DLP assay described above.

The test group received the CSPB protein solution at a dose level of 2160 mg/kg, the control group received the BSA protein solution at a similar dose level, and the dosing solutions were administered by oral gavage as two 17.5 ml/kg doses separated by approximately 4 h. Following dosing, all mice were subjected to detailed clinical observations and once daily thereafter for signs of mortality or toxicity. Food consumption was measured on days 0, 7 and 14. Body weights were measured prior to dosing and on study days 0, 7 and 14. All animals were sacrificed on day 14 and macroscopic exams were performed on the day of necropsy.

Data were statistically analyzed for homogeneity of variance using Levene's test followed by the Shapiro–Wilk test for normality. If both tests were not significant a single-factor parametric ANOVA followed by Dunnett's test was used to identify statistically significant differences between the control group and the test article-treated. If either test was significant a Kruskal–Wallis nonparametric ANOVA followed by Dunn's test was used to identify statistically significant differences between the control group and the test article-treated. The analyses were conducted with a minimum significance level of $p < 0.05$.

3. Results

3.1. Purification of CSPB

Initial screening demonstrated that *E. coli* harboring the CSPB expression plasmid was able to produce a high level of soluble CSPB, reaching up to 8% of the total soluble protein. Following the optimization of purification conditions, CSPB was purified from 5 kg of *E. coli* cell paste, resulting in 8 g of CSPB. A silver stained

SDS–PAGE gel revealed that CSPB was purified to 100% purity without any other proteins being detected (Fig. 1, lane 2).

The purification of CSPB from the grain of DroughtGard™ was a challenge due to the extremely low expression level of CSPB (~0.06 μg protein/g grain (USDA and Submission, 2009)). Thus, while the methods developed for the *E. coli*-produced CSPB purification served as the foundation for purification of CSPB from grain, significant additional process optimization was required. Grain extracts contain lipids, starch, and fibers, which complicate the purification process. CSPB extraction from the grain appeared to be optimal when performed under mildly basic conditions (~pH 8), low ionic strength buffers, and in the presence of a chelating agent, but with no other additives, detergents or denaturants. Diatomaceous earth also used in a filtration process which not only facilitated the filtration but also assisted in the removal of lipids and hydrophobic particles (Ward and Swiatek, 2009). The most critical step in the isolation process was employment of immunoaffinity chromatography to capture the very limited amount of CSPB present in the large volume of sample. To maximize yield at this step, the CSPB-enriched sample was recirculated over the immunoaffinity column multiple times to increase residence time and maximize the capture of CSPB.

The developed method allowed ~60 μg of CSPB to be obtained from 10 kg of DroughtGard™ grain, with a purity of ~97% (Fig. 1, lane 3). Recovery of the plant-produced CSPB during the purification process is summarized in Table 1. The low abundance of the protein in the starting material resulted in an approximately 50,000 fold purification of CSPB relative to its concentration in the starting extract (Table 1).

3.2. Characterization of CSPB

The identity of CSPB was confirmed using N-terminal sequencing (Table 2). N-terminal sequencing resulted in the expected sequences for CSPB with the exception of the N-terminal methionine, which was excised in the plant-produced CSPB (Table 2). Removal of the N-terminal methionine by methionine aminopeptidase is a common modification that occurs co-translationally before completion of the nascent protein chain, and typically has no effect on protein structure or activity (Arfin and Bradshaw, 1988; Bradshaw et al., 1998; Polevoda and Sherman, 2000).

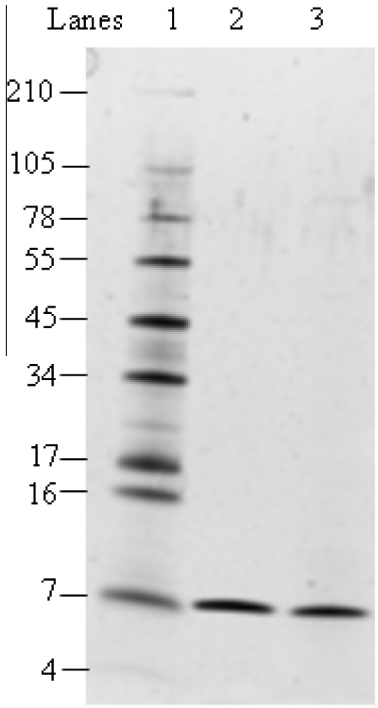

Fig. 1. SDS–PAGE of plant- and *E. coli*-produced CSPB. Aliquots of plant- and *E. coli*-produced CSPB were separated by SDS–PAGE on a Novex 10–20% (w/v) Tricine gel and stained with an Owl Silver Staining kit. Lane 1: molecular weight markers (values shown at left of image indicate molecular weight/1000), lane 2: the *E. coli*-produced CSPB, lane 3: the plant-produced CSPB.

The identity of plant- and *E. coli*-produced CSPB was further confirmed by tryptic peptide mass mapping using MALDI–TOF MS analysis. The identified masses were used to assemble a coverage map for the entire CSPB protein. The identified peptides from plant- and *E. coli*-produced CSPB resulted in 87.9% and 94% coverage of the entire protein sequence (Table 2, Fig. 2), respectively. This analysis confirmed the identity of both plant- and *E coli*-produced CSPB.

3.3. Evaluation of the equivalence between plant- and E. coli-produced CSPB

In order to use *E. coli*-produced protein to assess CSPB safety, it was important to establish the equivalence of this protein to the CSPB protein present in DroughtGard™ maize (Gao et al., 2004; Harrison et al., 1996; Raybould et al., 2013). Despite the very low expression level of CSPB in the grain of DroughtGard™ maize, a sufficient amount of highly purified plant-produced CSPB was obtained to enable this equivalence assessment. Immunoblot analysis was conducted to determine the relative immunoreactivities of plant- and *E. coli*-produced CSPBs. The results demonstrated that proteins from both sources migrated to a similar position on the blot (Fig. 3) and showed comparable band intensities. This analysis demonstrated that both proteins have equivalent immunoreactive properties.

The equivalence in apparent molecular weight of plant- and *E. coli*-produced CSPB was demonstrated using SDS–PAGE. The apparent molecular weights of plant- and *E. coli*-produced CSPB were 6.7 and 6.5 $\times$ 10^3 (Table 2), respectively, as assessed on separate SDS–PAGE gels. The difference in their apparent molecular weights was 3%, which is well within typical assay variability (Weber and Osborn, 1969). This analysis demonstrated that both proteins have equivalent apparent molecular weights.

The specific activity to unfold RNA was determined to be 0.660 ± 0.05 pmol opened DLP/μg of the plant-produced CSPB and 0.757 ± 0.055 pmol opened DLP/μg of the *E. coli*-produced CSPB (Table 2). The difference of specific activities between CSPB isolated from different sources was 12.8%, which was within acceptable assay variability (15%) determined during the assay validation, confirming that plant- and *E. coli*-produced CSPB have equivalent functional activities. This result also indicates that the N-terminal methionine, absent in the plant-produced protein, did not affect CSPB functional activity.

Some eukaryotic proteins are post-translationally modified by the addition of carbohydrate moieties (Rademacher et al., 1988). The search for glycosylation sites found two potential sites in the CSPB protein: one O linked at T64 and one N-linked at N62 (see Fig. 2B for amino acid positions). Because glycosylation can alter the physiochemical properties of a protein (e.g., function, half-life), assessing whether the plant-produced protein is glycosylated is one component of the equivalence assessment (Raybould et al., 2013). CSPB isolated from the grain and *E. coli* were each evaluated for glycosylation, using transferrin and horseradish peroxidase (HRP) as positive controls. The positive controls, transferrin and HRP, were detected at the expected molecular weights of ~75 $\times$ 10^3 and ~50 $\times$ 10^3, respectively, in a concentration-dependent manner (Fig. 4A, lanes 4–5 and 2–3). No detectable signal was observed for the plant-produced CSPB (Fig. 4A, lanes 6 and 7) and CSPB from *E. coli* (Fig. 4A, lanes 8 and 9). To confirm that

Table 1
Recoveries of the plant-produced CSPB during purification process.

Samples	Total protein (g)	CSPB (μg)[a]	CSPB per total protein (μg/g)	Purification fold[b]
Clarified extract	34.5	713	20.7	–
Diafiltered sample	37.5	737.1	19.7	1
Ammonium sulfate cut	19.6	725.9	37.1	1.8
Desalted sample	15.4	602	39.1	1.9
AEC pool	6.3	529.7	84.1	4.1
IAC/SEC pool	N.A.	102.9	N.A.	N.A.
Purified CSPB	6.1×10^{-5}	59	0.97×10^{6}	46,860

Note: AEC, anion exchange chromatography; IAC, immunoaffinity chromatography; SEC, size exclusion chromatography; N.A., not available.
[a] Assayed by enzyme-linked immunosorbent assay with exception of purified CSPB sample, which was determined by densitometric scanning of an SDS–PAGE visualized by silver staining.
[b] Purification fold equals CSPB per total protein divided by the starting amount (20.7).

Table 2
Characteristics of plant- and *E. coli*-produced CSPB.

Characteristic	Method	Result	
		E. coli-produced	Plant-produced
Purity	SDS–PAGE/densitometry	100%[a]	97%[b]
Apparent molecular weight	SDS–PAGE/densitometry	6.5×10^{3}[a]	6.7×10^{3}[b]
Activity	DNA unfolding[c]	0.757 ± 0.055 U	0.660 ± 0.05 U
Immunoreaction	Immunoblot	Immunoreactive band observed	Equivalent immunoreactive band observed
Glycosylation	Molecular Probes' Pro-Q® Emerald 488 Glycoprotein Blot Stain Kit	Not glycosylated	Not glycosylated
Identity	N-terminal sequence	MVEGKVKWFNSEKGF	VEGKVKWFNSEKGFG
	MALDI–TOF MS (tryptic mass fingerprint)	94.0% coverage of expected sequence	87.9% coverage of expected sequence

[a] Proteins were visualized by colloidal blue staining and calculated as the average of the six loads.
[b] Proteins were visualized by silver staining and calculated as the average of the six loads.
[c] 1 U equals 1 pmol opened DLP/μg CSPB.

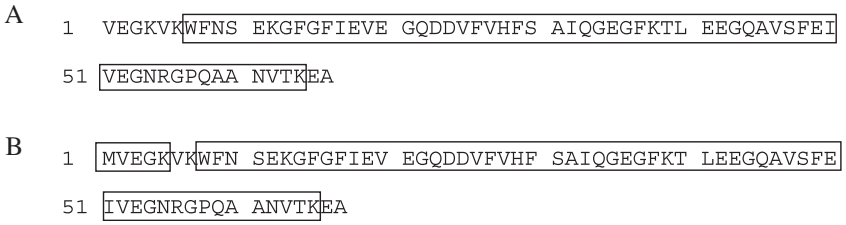

Fig. 2. MALDI–TOF MS coverage map of plant- and *E. coli*-produced CSPB. The amino acid sequence of the CSPB was deduced from the *cspb* gene present in DroughtGard™ maize. Boxed regions correspond to tryptic peptides that were identified from the plant- (A) and *E. coli*-produced CSPB (B) using MALDI–TOF MS.

sufficient plant- and *E. coli*-produced CSPB were present on the blot, the same PVDF membrane was incubated with SYPRO Ruby stain to detect proteins (Fig. 4B). Both plant- and *E. coli*-produced CSPB were clearly visible on the membrane (Fig. 4B, lanes 6–9). Transferrin and HRP showed weak signals upon SYPRO Ruby staining, possibly due to their less sensitivity to the staining. Nonetheless, both transferrin and HRP responded well to glycosylation staining.

N-glycosylation occurs in the secretory pathway and is well conserved in animals, plants, fungi, and social amoebae (Bushey et al., 2014). However, the CSPB protein is not targeted for the secretory pathway, supporting that CSPB is not N-glycosylated. In addition, the tryptic fragments containing potential glycosylation sites were identified for both *E. coli* and plant-produced CSPB by MALDI–TOF mass spectrometry. All identified masses matched the expected non-modified peptide masses (Fig. 2), confirming that no glycosylation had occurred.

3.4. Bioinformatics analyses of potential CSPB allergenicity and toxicity

A key element of the safety evaluation for introduced proteins in biotechnology-derived crops is a bioinformatic analysis to assess whether the introduced protein shows similarity to known toxins or allergens (Hammond et al., 2013; Kier and Petrick, 2008). Bioinformatics analyses were performed on the translated sequence of the *cspb* coding sequence in DroughtGard™ to assess its structural relatedness with allergens, toxins and other relevant biologically active proteins. All alignments were thoroughly evaluated according to criteria described in the Methods. The results of the search comparisons showed that no relevant alignments were observed against proteins in the allergen database (FARRP, 2014). Likewise, no relevant alignment was observed with the translated open reading frame derived from *cspb* as compared to proteins in the protein toxin database. These data demonstrate the lack of sequence similarities between the CSPB sequence and any allergenic, toxic or biologically harmful proteins. To evaluate the similarity of CSPB to known proteins, a FASTA search using CSPB as a query to search GenBank release 199 was also conducted and resulted in self identification. Of the top 10,000 alignments, greater than 6600 were with proteins that were described as being "cold shock". Among the organisms that contain an aligning cold shock protein was *Lactobacillus bulgaricus*, an organism used in the production of yogurt. The remaining alignments included patent sequences

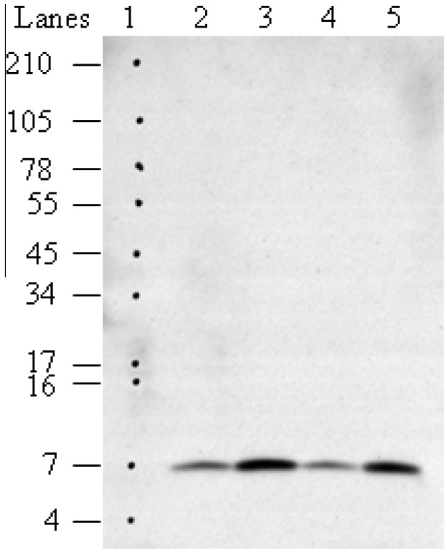

Fig. 3. Immunoblot analysis of plant- and *E. coli*- produced CSPB. Aliquots of plant- and *E. coli*-produced CSPB were separated by SDS–PAGE on a Novex 10–20% (w/v) Tricine gel, and electrotransferred to a nitrocellulose membrane (pore size: 0.45 μm). The blot was probed with goat anti-CSPB specific polyclonal antibodies and developed using an Enhanced chemiluminescence system. Lane 1: molecular weight markers (values shown at left of image indicate molecular weight/1000), lanes 2 and 3: the *E. coli*-produced CSPB, lanes 4 and 5: the plant-produced CSPB, lanes 2 and 4: 2 ng, lanes 3 and 5: 5 ng.

and translations of hypothetical gene sequences from genome sequencing projects.

3.5. Assessment of CSPB susceptibility to digestive enzymes

An assessment of CSPB susceptibility to pepsin was conducted using a standardized assay (Thomas et al., 2004). Cleavage of the CSPB protein by pepsin was evaluated by visual analysis of stained polyacrylamide gel (Fig. 5A). The limit of detection (LOD) of the CSPB protein by Colloidal Brilliant Blue G staining was observed at approximately 0.005 μg or approximately 0.6% of the total CSPB protein loaded (Supplemental Fig. 1). Western blot analysis was also conducted (Supplemental Fig. 2); the LOD of the CSPB protein by western blotting was observed at approximately 0.1 ng or approximately 1% of the total CSPB protein loaded (Supplemental Fig. 3). Visual examination of a stained gel (Fig. 5A) showed that the full-length CSPB protein was degraded below LOD within 0.5 min of exposure to pepsin (Fig. 5A, lane 5). Therefore, based on the LOD, at least 99% of the CSPB protein was degraded by pepsin within 0.5 min. A transiently-stable protein fragment with an apparent molecular weight of $\sim 2.5 \times 10^3$ was observed between the 0.5 and 30 min time points. The relative abundance of the transient fragment to the CSPB protein initially loaded was estimated by densitometry. At the 10 and 30 min time points, the transient fragment band had an intensity of 11.8% and 2.6% of the intact CSPB protein found at the 0 min time point, respectively. This fragment was derived from the N-terminus of the CSPB protein (data not shown). No CSPB fragments were visible at the 60 min time point (Fig. 5, lane 11) indicating complete degradation of the protein by pepsin.

To better understand the degradation fate of the transiently stable CSPB fragment, an assessment of the susceptibility of this fragment to pancreatin and neutral pH was conducted (Fig. 5B). After CSPB treatment with pepsin for 2 min, the reaction was quenched and the transiently stable $\sim 2.5 \times 10^3$ CSPB protein fragment was exposed to pancreatin. The cleavage of the CSPB-derived fragment by pancreatin was evaluated by SDS–PAGE (Fig. 5B). Visual examination of the stained gel demonstrated that this fragment was degraded by pancreatin within 0.5 min, the first time point assessed (Fig. 5B, lane 7).

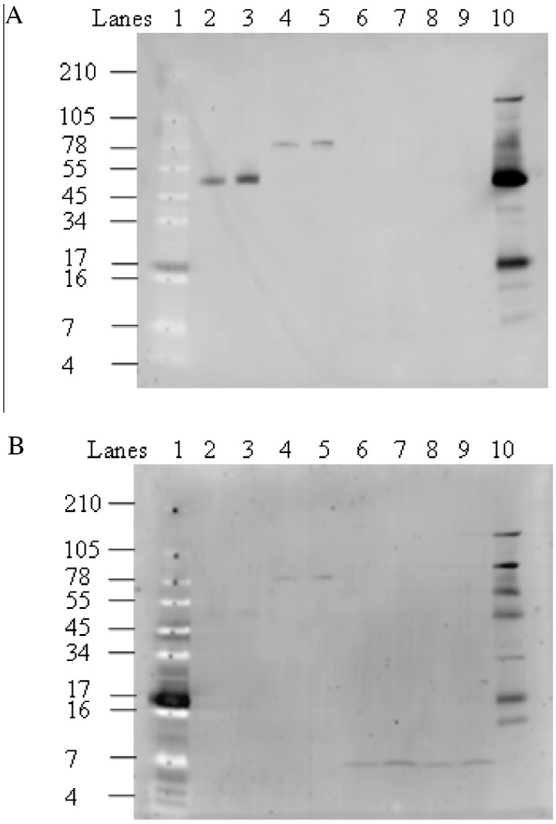

Fig. 4. Glycosylation analysis of CSPB. Aliquots of plant- and *E. coli*-produced CSPB, HRP (positive control) and transferrin (positive control) were separated by SDS–PAGE on a Novex 10–20% (w/v) Tricine gel and electrotransferred to a PVDF membrane (pore size: 0.45 μm). (A) Where present, periodate-oxidized protein-bound carbohydrate moieties reacted with Pro-Q Emerald 488 glycoprotein stain and emitted a fluorescent signal at 488 nm (lanes 2–5 and 10). (B) The same blot was stained with SYPRO Ruby to demonstrate the protein loading. The signal was captured using a Bio-Rad Molecular Imager FX. Lane 1: molecular weight markers (values shown at left of image indicate molecular weight/1000); lanes 2 and 3: HRP; lanes 4 and 5: transferrin; lanes 6 and 7: the plant-produced CSPB; lanes 8 and 9: the *E. coli*-produced CSPB; lane 10: CandyCane glycosylated protein markers; lanes 2, 4, 6 and 8: 25 ng; lanes 3, 5, 7 and 9: 50 ng.

3.6. Assessment of potential oral toxicity of CSPB

In addition to the above data, demonstrating that CSPB is not a hazard, further confirmation of safety was demonstrated with an acute toxicity test for the CSPB protein. The test group mice were dosed at 2160 mg CSPB/kg body weight. A dose level of BSA comparable to the CSPB dose levels served as the control formulation. The dose formulation was analyzed for CSPB concentration, homogeneity, stability and activity and met all of the acceptability criteria for those parameters (data not shown). The animals were dosed on Day 0 and observed for 14 additional days. All animals were healthy, and no mortality or CSPB-related clinical signs were observed. CSPB did not affect body weight (Table 3), or food consumption (Table 4), and no abnormal gross necropsy findings were present at study completion. For the purposes of assessing dietary exposure, the no observable adverse effect levels (NOAEL) for CSPB was considered to be 2160 mg/kg for the study.

3.7. Assessment of dietary exposure to CSPB

To estimate dietary exposure to CSPB, the expression level of the protein in DroughtGard™ grain and the consumption of maize

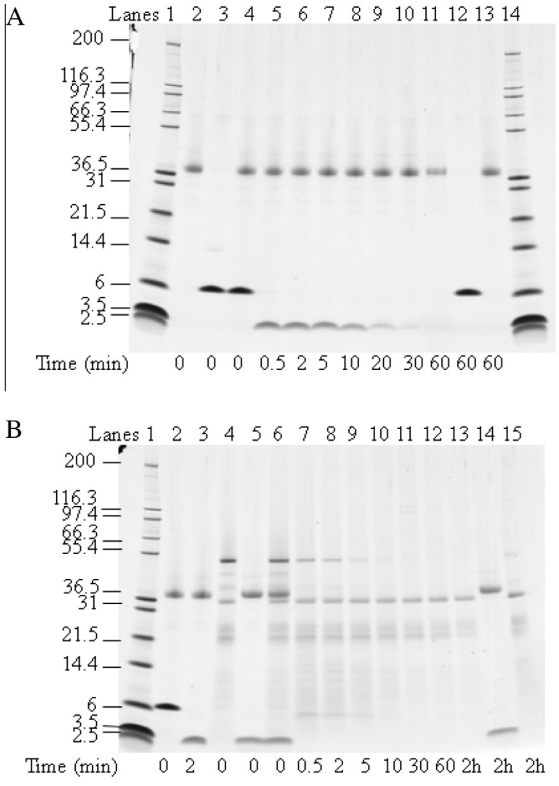

Fig. 5. Colloidal Brilliant Blue G stained SDS-gels of CSPB protein digestion. Panel A corresponds to CSPB protein digestion by pepsin. Based on pre-digestion protein concentrations, 0.8 μg CSPB protein was loaded in lanes containing CSPB protein. The incubation times are indicated. Lanes 1 and 14: molecular weight markers (values shown at left of image indicate molecular weight/1000), lanes 2 and 13: pepsin only, lanes 3 and 12: CSPB only, lanes 4–11: a time course of pepsin digestion. Panel B: After digestion of the CSPB protein by pepsin for 2 min, the reaction was quenched and the transiently stable CSPB protein fragment was exposed to further digestion by pancreatin. Based on protein concentrations before digestion by pepsin, 0.8 μg of total protein was loaded per lane containing CSPB protein. Lanes 1: molecular weight markers (values shown at left of image indicate molecular weight/1000), lane 2: pepsin reaction mixture, lane 3: 2 min after digestion of CSPB by pepsin, lanes 4 and 15: pancreatin mixture only, lanes 5 and 14: no pancreatin added to reaction, lanes 6–13: a time course of pancreatin digestion. 2 h, 2 hours.

and maize-derived products were determined. The average CSPB expression level in DroughtGard™ grain grown in the U.S. during 2006 was 0.063 μg/g fresh weight (USDA and Submission, 2009). This protein expression estimate is a conservative estimate of CSPB concentration in food derived from DroughtGard™ maize because it assumes no loss of the CSPB protein during storage, processing and/or cooking of the grain or food. The highest 97.5th percentile consumption estimates for acute (single-day "eaters only") consumption of maize and maize-derived products were according to the GEMS/Food database (WHO, 2011). These values were selected because they are the highest reported values world-wide and represent a reasonable estimate of the maximum amount of maize that might be consumed in a single day. The acute consumption of maize flour in the general population and children aged six years and younger was derived from food consumption surveys in France and Australia, respectively. The consumption values in the general population and children aged six for sweet corn and popcorn were derived from food consumption surveys in Thailand and Japan, respectively. Additionally, most maize products, with the exception of maize on the cob, are blended commodities. This makes it very difficult to estimate the percentage of consumed maize products that will be derived from DroughtGard™. As a result, the conservative assumption was made that 100% of all maize products consumed would be derived from DroughtGard™. As one of many commercial maize varieties currently grown, DroughtGard™ would represent only a small fraction of the maize used to prepare maize food products. Based on these high-level consumption values and the level of expression of CSPB in DroughtGard™ maize grain, the dietary intakes of CSPB from these maize and maize-derived products were calculated and are shown in Table 5. These CSPB intake values were then used to calculate the margin of exposure (MOE) for acute dietary intake of CSPB from maize and maize products containing DroughtGard™ compared to acute toxicity study NOAEL of 2160 mg/kg (Table 5), which was the highest dose tested. The results indicate very large MOEs that are $\geqslant 3.0 \times 10^6$ even when human exposure to CSPB is generously estimated due to the conservative assumptions made in the exposure assessment.

4. Discussion

An essential component of the safety assessment of biotechnology-derived crops is evaluation of the safety of newly expressed proteins (Delaney et al., 2008a; Hammond et al., 2013; Herouet et al., 2005; Kier and Petrick, 2008). This comprehensive safety assessment includes: (1) the evaluation of the history of safe use of the donor organism and the protein itself, (2) examination of the mechanism of action of the introduced protein, (3) examination

Table 3
Summary of body weight data observed in the acute oral toxicity study in mice.

Group	Time[a]									
	Day 0		Day 7		Change (day 0 to day 7)		Day 14		Change (day 0 to day 14)	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
CSPB	31.2 (1.75)	23.4 (1.03)	34.1 (2.73)	25.6 (1.21)	2.9 (1.55)	2.2 (1.07)	35.6 (3.35)	26.9 (1.29)	4.4 (2.13)	3.5 (0.99)
BSA	31.4 (1.42)	23.5 (1.15)	34.0 (1.45)	25.1 (1.63)	2.6 (1.07)	1.6 (1.33)	36.4 (1.60)	27.4 (1.60)	5.1 (1.68)	3.9 (0.88)

[a] Values correspond to the mean body weight (and standard deviation) of 10 animals in units of grams.

Table 4
Summary of food consumption data observed in the acute oral toxicity study in mice.

Group	Time[a]			
	Change (day 0 to day 7) g animal^{-1} day^{-1}		Change (day 7 to day 14) g animal^{-1} day^{-1}	
	Male	Female	Male	Female
CSPB	7.0 (1.60)	6.1 (0.75)	7.2 (1.57)	6.3 (0.66)
BSA	7.3 (1.78)	5.9 (0.41)	7.0 (0.46)	6.3 (1.00)

[a] Values correspond to the mean (and standard deviation) of 10 animals per group.

Table 5
Risk assessment/margin of exposure.

Population	Consumption of food item[a] (g/kg/day)	CSPB intake[b] (μg/kg/day)	Margin of exposure[c]
Maize flour			
General population (97.5th percentile "eater-only", acute value)	2.04	0.129	1.7×10^7
Children ⩽ 6 years (97.5th percentile "eater-only", acute value)	3.16	0.199	1.1×10^7
Popcorn			
General population (97.5th percentile "eater-only", acute value)	3.33	0.210	1.0×10^7
Children ⩽ 6 years (97.5th percentile "eater-only", acute value)	3.33	0.210	1.0×10^7
Sweet corn			
General population (97.5th percentile "eater-only", acute value)	7.16	0.451	4.8×10^6
Children ⩽ 6 years (97.5th percentile "eater-only", acute value)	11.52	0.726	3.0×10^6

[a] Estimates of acute consumption were obtained from the WHO Global Environmental Monitoring System (WHO, 2011).
[b] Assumes 100% of maize-derived food was derived from DroughtGard™ maize and contains 0.063 μg/g (ppm) CSPB (USDA and Submission, 2009).
[c] Calculated by dividing NOAEL from acute mouse gavage study (2160 mg/kg/day) by CSPB intake. MOEs rounded to two significant digits.

of the similarity of the protein to known allergens and toxins, (4) characterization of the physicochemical and functional properties of the protein, (5) evaluation of the digestibility of the protein by pepsin, (6) a toxicity assessment, and (7) an intake assessment.

CSPB is derived from *B. subtilis*, a microorganism with a history of safe use in fermented foods and probiotics (De Boer and Diderichsen, 1991; Hosoi et al., 2003; Sanders et al., 2003). *B. subtilis* has been used in production of food for hundreds of years (Ashikaga et al., 2000; FDA, 1999; Inatsu et al., 2006; OECD, 2001). In addition, *B. subtilis* was tested for cytotoxicity in Chinese hamster ovary K1 (CHO-K1) cells, for production of hemolytic and nonhemolytic enterotoxins (Pedersen et al., 2002), acute toxicity in BALB/c mice, and chronic toxicity in mice, rabbits, and pigs (Sorokulova et al., 2008). No toxic effects were attributed to *B. subtilis* in these studies and no similarities to pathogenic bacteria were found, which led to the conclusion that *B. subtilis*, the donor organism for CSPB, is safe for human consumption (Pedersen et al., 2002; Sorokulova et al., 2008).

CSPB is similar to other CSPs and related proteins found in many bacteria and plants, including those used for food (Graumann and Marahiel, 1999; Karlson and Imai, 2003; Nakaminami et al., 2006). This family includes bacterial and plant proteins possessing a CSD that has high amino acid sequence similarity to the sequence of the CSPB protein (Graumann et al., 1997; Karlson and Imai, 2003; Nakaminami et al., 2006). CSPB from *B. subtilis* is homologous to the CSP proteins found in *Lactobacillus*, *Lactococcous*, and *Bifidobacterium* species, the most common types of bacteria used in the dairy industry to prepare cheese, sour cream, buttermilk, yogurt and probiotic products containing live bacterial cultures (Morea et al., 2001; Ogier et al., 2002). Plant proteins with CSDs that have high similarity to the CSPB protein are present in many crops. Thus, the CSPB protein expressed in DroughtGard™ maize is similar to several bacterial CSPs and CSD-containing proteins present in the human diet and directly consumed in common foods, establishing a history of safe exposure for this protein.

Plant CSD proteins, like bacterial CSPs, accumulate in response to cold (Fusaro et al., 2007; Nakaminami et al., 2006, 2005), and exhibit *in vitro* nucleic acid binding activity with a low sequence specificity. The CSD-proteins have been shown to bind and destabilize RNA secondary structures (Sasaki et al., 2007), suggesting that CSD proteins regulate stress responses in plants through mechanisms similar to those described for CSP proteins in bacteria. A plant CSD protein has been shown to rescue a cold-sensitive *E. coli* CSP knockout mutant (Nakaminami et al., 2006), indicating conserved functions between bacterial CSP and plant CSD-containing proteins. The transgenic maize plants expressing the CSPB protein demonstrated yield improvement under water-limited conditions (Castiglioni et al., 2008). However, disruption of the nucleic acid binding activity of CSPB by single amino acid mutation abolished the ability of the CSPB protein to confer maize yield benefits under water stress (Castiglioni et al., 2008). The loss of drought tolerance phenotype by expression of the CSPB functional mutant indicated that CSPB likely moderates drought responses in DroughtGard™ maize through a similar mechanism to CSD-containing proteins in plants, possibly through destabilization of RNA secondary structures. This conserved function between CSPB in DroughtGard™ maize and CSD-containing proteins in plant supports a lack of a safety concern with respect to CSPB's mechanism of action.

Bioinformatic analysis of amino acid sequences addresses a fundamental safety question: does the introduced protein share any sequence or structural similarity with proteins known to pose possible hazards? Because high sequence and structural similarity often correlates with a conserved biological role, bioinformatic analysis provides an important assessment of the potential for protein toxicity or allergenicity (Hammond et al., 2013). The analyses demonstrated that no structurally relevant similarity exists between CSPB and known toxic, allergenic, or other biologically active proteins that would be harmful to human or animal health. An absence of homology between CSPB and known allergens and protein toxins supports the conclusion that CSPB is not a hazard.

While both the history of safe use and bioinformatics analyses indicate that the consumption of the CSPB protein is safe, additional safety studies were conducted because it is the first known RNA-unfolding protein used in the context of agricultural biotechnology. A challenge for the safety assessment of proteins expressed in crops is producing enough high quality protein to perform safety assessments. Because the expression of CSPB in grain of DroughtGard™ is very low, it is not technically feasible to obtain sufficient quantities of CSPB for safety assessment from grain. In such circumstances, it is acceptable for the needed quantity of the protein to be produced from an alternative source, such as *E. coli* (Gao et al., 2004; Harrison et al., 1996; Herouet et al., 2005). However, a small amount of protein must still be purified from the plant to assess its equivalence to the *E. coli*-produced protein. In this study, both plant- and *E. coli*-produced CSPB were physicochemically and functionally characterized, and the equivalence between them was demonstrated according to Codex and other published guidelines (Codex Alimentarius, 2009; Gao et al., 2004; Harrison et al., 1996; Herouet et al., 2005; Raybould et al., 2013). Accordingly, the *E. coli*-produced CSPB protein is considered suitable for conducting safety assessments of the CSPB protein present in DroughtGard™.

Although the correlation between allergenicity and pepsin resistance is imperfect (Herman et al., 2006), some proteins that are food allergens are relatively resistant to pepsin digestion (Asero et al., 2000; Astwood et al., 1996; Yagami et al., 2000). As a result, evaluation of the susceptibility of the protein of interest to digestion by the gastric enzyme pepsin is an important element of the safety assessment for introduced proteins (Codex

Alimentarius, 2009). In addition, rapid degradation by pepsin provides evidence that exposure to the introduced protein will be minimized following consumption (Hammond et al., 2013; Kier and Petrick, 2008). The full length CSPB was rapidly degraded by pepsin (none detectable after 0.5 min incubation), and a small ($\sim 2.5 \times 10^3$) transiently stable fragment was completely degraded after 60 min by pepsin. During human gastrointestinal digestion, protein or protein fragment(s) must first pass through the stomach where they are exposed to pepsin and then the duodenum where they are exposed to pancreatin. The pepsin-released CSPB fragment is less than 3×10^3 and is only transiently present during pepsin digestion. Therefore, it is unlikely that such fragment will pose a potential allergenic risk (Ofori-Anti et al., 2008). However, in instances where transient stability of a protein or protein fragment is observed in pepsin cleavage, sequential degradation of the fragment by pepsin followed by pancreatin has been recommended (Bushey et al., 2014; Delaney et al., 2008a). For that reason, an assessment of the susceptibility of this CSPB fragment to pancreatin was conducted. It was observed that this fragment is rapidly degraded by pancreatin within 0.5 min. Thus, CSPB is highly susceptible to mammalian digestive enzymes, a result consistent with the conclusion that CSPB is safe for consumption.

As discussed above, CSPB protein (1) has a history of safe use; (2) is not structurally or functionally related to known toxins or allergens; (3) has a nucleic acid binding activity conserved in the CSD family of proteins; (4) is readily cleaved by mammalian digestive enzymes; and (5) has a very low expression level in DroughtGard™. Thus, the weight of evidence supports that CSPB is not a hazard to people (Hammond et al., 2013). However, experimental evaluation of potential toxicity of CSPB was still conducted to further assess the safety of CSPB. An acute toxicity study is the appropriate test to examine that CSPB is not toxic (Pariza and Johnson, 2001), because most known protein toxins exert toxicity through acute mechanisms (Pariza and Johnson, 2001; Sjoblad et al., 1992).

The NOAEL is the dose that causes no adverse effects in test animals, and is used to establish a safe level of exposure for humans. A protein toxicity test, with a dose of 2160 mg CSPB/kg body weight, resulted in no adverse impacts on the treatment animals and established 2160 mg CSPB/kg as the NOAEL. This NOAEL and estimates of dietary intake of CSPB were used to calculate MOEs for CSPB in maize and maize products that could be derived from DroughtGard™ (i.e., 100% of maize consumed is DroughtGard™ and no CSPB is lost due to storage, processing, or cooking). All MOEs were $\geqslant 3 \times 10^6$, or more than six orders of magnitude higher than generous estimates for human exposure to CSPB from consumption of DroughtGard™ maize. Moreover, given the low expression of CSPB in DroughtGard™ maize (average 0.063 μg protein/g) a 60 kg human would have to eat approximately 2.1×10^6 kg of DroughtGard™ maize in a single day to achieve an intake level similar to the NOAEL of the acute toxicity study (2160 mg/kg). These results indicate that there are no meaningful risks to human health from dietary exposure to the CSPB protein derived from DroughtGard™.

Acute toxicity studies of introduced proteins may be conducted in mice at a high dose of 2000 mg/kg via oral gavage, the dose of which is designed for testing of chemicals (OECD, 2001). However, the CSPB dose level tested in this case becomes irrelevant to a realistic dietary exposure as the MOE was $\geqslant 3 \times 10^6$.

5. Conclusions

As part of the safety assessment of GM crops, the introduced protein was characterized and assessed in a battery of tests. These analyses included an evaluation of the history of safe use of the CSPB protein, bioinformatics analyses of the similarity of CSPB to known allergens, toxins, and anti-nutritional proteins, an assessment of cleavage by mammalian digestive enzymes, acute toxicity testing of the CSPB protein and an assessment of consumption of CSPB from DroughtGard™ maize. The outcome of these analyses support the conclusion that the CSPB expressed in DroughtGard™ is safe for consumption in food and feed.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.yrtph.2014.12.014.

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